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Nutrient optimization of tree growth alters structure and function of boreal soil food webs

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ABSTRACT

Nutrient optimization has been proposed as a way to increase boreal forest production, and involves chronic additions of liquid fertilizer with amounts of micro- and macro-nutrients adjusted annually to match tree nutritional requirements. We used a short-term (maintained since 2007) and a long-term (maintained since 1987) fertilization experiment in northern Sweden, in order to understand nutrient optimization effects on soil microbiota and mesofauna, and to explore the relationships between plant litter and microbial elemental stoichiometry. Soil microbes, soil fauna, and aboveground litter were collected from the control plots, and short- and long-term nutrient optimization plots. Correlation analyses revealed no relationships between microbial biomass and litter nutrient ratios. Litter C:N, C:P and N:P ratios declined in response to both optimization treatments; while only microbial C:P ratios declined in response to long-term nutrient optimization. Further, we found that both short- and long-term optimization treatments decreased total microbial, fungal, and bacterial PLFA biomass and shifted the microbial community structure towards a lower fungi:bacterial ratio. In contrast, abundances of most fungal- and bacterial-feeding soil biota were little affected by the nutrient optimization treatments. However, abundance of hemi-edaphic Collembola declined in response to the long-term nutrient optimization treatment. The relative abundances (%) of fungal-feeding and plant-feeding nematodes, respectively, declined and increased in response to both short-term and long-term treatments; bacterial-feeding nematodes increased relative to fungal feeders. Overall, our results demonstrate that long-term nutrient optimization aiming to increase forest production decreases litter C:N, C:P and N:P ratios, microbial C:P ratios and fungal biomass, whereas higher trophic levels are less affected.

1. Introduction

Boreal forest growth is generally limited by nitrogen (N), while limitation by other nutrients such as phosphorus (P) and potassium (K) become increasingly important when N is supplied (Tamm, 1991; Tanner et al., 1998; Tripler et al., 2006). In order to enhance timber production and increase carbon (C) sequestration, alleviating nutrient limitation in boreal forests is common (Beringer et al., 2011; Fernández-Martínez et al., 2014). In general, increased nutrient availability *via* fertilization enhances photosynthetic rate, foliar nutrient content, and tree growth and C accumulation (Bergh et al., 1999; From et al., 2016; Niu et al., 2016). In Scandinavian forestry, fertilizers are commonly applied as a single dose or as repeated applications of solid N fertilizer (*ca.* 150 kg N ha^{-1}) (Bergh et al., 2005; From et al., 2015). However, solid N fertilization can also have detrimental effects on boreal forest ecosystems, such as compositional shifts in understory vegetation, nutrient imbalances, nutrient toxicity effects, nutrient leaching, as well as shifts in soil community structure and functioning (Binkley and Högberg, 2016; Gundale et al., 2011; Lucas et al., 2011; Strengbom and Nordin, 2008; Treseder, 2008).

As an alternative to solid N fertilization, the nutrient optimization approach entails the chronic addition of liquid fertilizer containing

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micro- (Mn, Fe, Zn, Cu, B and Mo) and macro-nutrients (N, P, K, Ca, S and Mg) through an irrigation system. This may reduce some of the detrimental effects mentioned above for N fertilization (*e.g.* Fransson et al., 2000). So far, most nutrient optimization studies in boreal ecosystems have focused on aboveground organisms, reporting a decline of understory vegetation of the most common plant groups, *i.e.* bryophytes and ericaceous dwarf shrubs (Hedwall et al., 2013; Strengbom et al., 2011). Changes in understory vegetation may in turn affect the structure and functioning of aboveground food webs, specifically herbivores and herbivore-predator interactions (Meunier et al., 2015; Throop and Lerdau, 2004). Despite plant litter serving as primary C resource for soil decomposers, few studies have investigated how nutrient optimization impacts litter-mediated effects on soil biota and their trophic interactions (Lindberg and Persson, 2004).

In addition to changing the quantity or quality of organic inputs (*e.g.* plant litter, mycorrhizal C transfer, and root exudates), fertilization may also alter the chemistry of organic matter, that subsequently impacts the activity and community structure of primary consumers (*i.e.* microbes; Bokhorst et al., 2017; Meunier et al., 2015; Wallenda and Kottke, 1998). For example, Maaroufi et al. (2015) reported a decline of fungal biomass in forest plots where half of the total N added over 16 years of N fertilization was retained in the soil humus, thereby reducing the humus C:N ratio. Similar patterns have been reported in other nutrient optimization studies (Demoling et al., 2008; Fransson et al., 2000). While these studies demonstrate impacts of nutrient enrichment on soil microbial biomass, nutrient enrichment may also impact microbial C:N:P stoichiometry. This is of great interest because changes in microbial elemental stoichiometry may control key soil processes such as nutrient retention and losses (Zechmeister-Boltenstern et al., 2015). It has been proposed that globally the C:N:P stoichiometry of microbial communities is very stable and independent of the environmental nutrient availability (Cleveland and Liptzin, 2007; Mooshammer et al., 2014). However, other studies have challenged this view by showing that microbial community C:N:P stoichiometry can vary with its surrounding environment (*e.g.* soil and soluble litter fractions) (Fanin et al., 2013; Tischer et al., 2014). Thus, it remains poorly understood to what degree nutrient optimization affects soil microbial stoichiometry.

Changes in microbial abundance and stoichiometry may further affect soil fauna at higher trophic levels of the soil food web (see Fig. 1). Soil fauna are of interest because they regulate ecosystem processes involved in organic matter turnover and associated nutrient mineralization (Bardgett and Wardle, 2010). Soil organisms may respond positively or negatively to nutrient addition in forest ecosystems. For example, long-term N addition studies have shown a decline of fungal-feeding and predaceous soil fauna in northern and sub-alpine forests (Gan et al., 2013; Xu et al., 2009). The few nutrient optimization studies focusing on soil food webs have also shown mixed effects on soil fauna. For example, after 10 years of nutrient optimization, Berch et al. (2009) did not find any effects on fungal-feeding Collembola but an increase of fungal-feeding mites. In contrast, Lindberg and Persson (2004) found no impact on fungal-feeding mites but an increase of Collembola after 12 years of nutrient optimization. In boreal forests, the main decomposition pathway is generally fungal-based, consisting of fungal biomass, fungivores and their predators. Previous studies have reported a decline of fungal decomposers and their activity in response to N fertilization relative to bacteria (Treseder, 2008). Thus, the relative importance of fungal *versus* bacterial decomposition pathways (also referred to as energy channels) might be also altered by nutrient optimization.

Here, we made use of a long-term fertilization experiment, maintained since 1987 at Flakaliden in northern Sweden, to characterize the ecological consequences of nutrient optimization on soil biota. Specifically, we studied the effect of short-term (6 years) and long-term

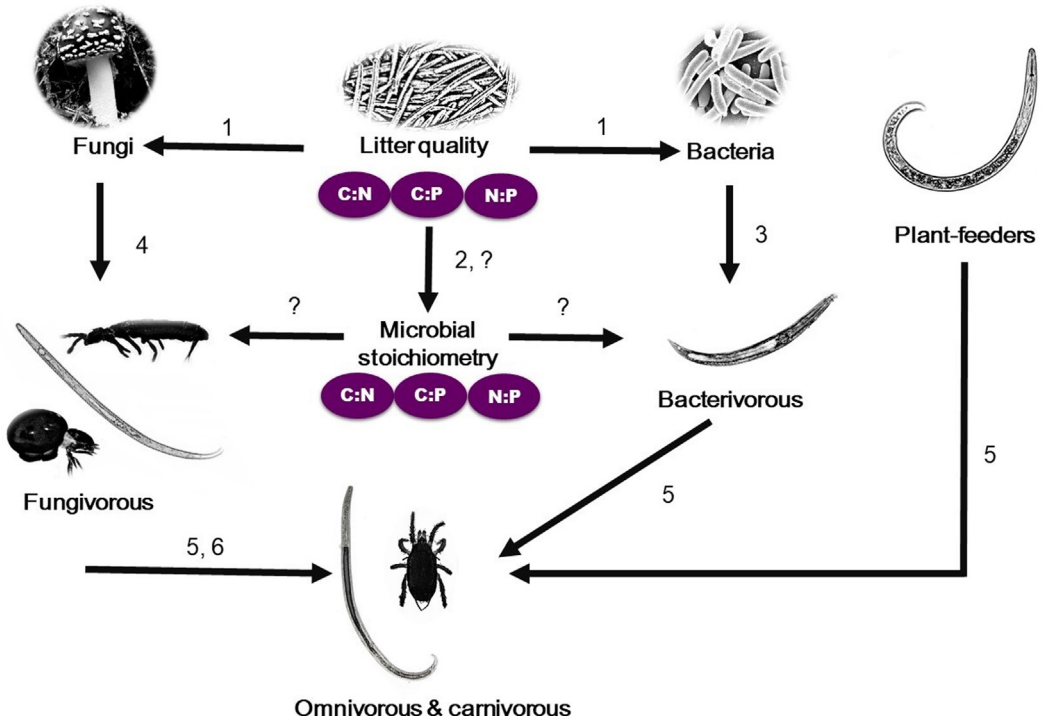

Fig. 1. Conceptual model showing the potential effects of litter and microbial elemental stoichiometry on soil food webs. Arrow numbers indicate different pathways and their associated mechanisms. Question marks indicate unknown or not well established relationships that were tested in this study. Litter quality may influence the primary consumers (bacteria and fungi) as aboveground litter represents one of the basal resources for the microbiota (1) (Coleman et al., 2004); litter quality may influence microbial stoichiometry (2; ?) (Tischer et al., 2014); bacteria are the primary food resource of bacterial-feeding nematodes (3) (Yeates et al., 1993); fungi are the primary food resource of fungal-feeding mites, fungal-feeding nematodes and Collembola (4) (Chahartaghi et al., 2005; Coleman et al., 2004; Yeates et al., 1993); nematodes are food resources for omnivorous and carnivorous nematodes (5) (Yeates et al., 1993); fungal-feeding mites, Collembola and nematodes are food resources for predatory mites (6) (Klarner et al., 2013).

(26 years) nutrient optimization on aboveground litter resource quality, microbial stoichiometry, and soil food web structure. We tested the following three hypotheses: (1) nutrient optimization will reduce bacterial and fungal biomass per unit soil mass and shift the microbial community structure towards a lower fungi:bacteria ratio, especially in the long-term treatment. (2) Decline in plant litter C:N and C:P ratios will reduce microbial biomass C:N and C:P ratios, particularly in the long-term treatment. (3) The decline of the primary consumer biomass (fungi and bacteria) will coincide with a shift in the relative importance of energy channels such that soil food web structure will shift towards more bacterial-dominated decomposition pathways. Specifically, we predict that differential impacts of nutrient optimization on fungi *versus* bacteria will affect bacterial and fungal consumers in contrasting ways: bacterial consumers (*i.e.* bacterial-feeding nematodes) will benefit, while fungal consumers (*i.e.* fungal-feeding nematodes, oribatid mites and springtails) will suffer from the nutrient optimization treatments. Taken together, our findings will improve our understanding of the impact of nutrient optimization on the structure and the function of soil food webs.

2. Material and methods

2.1. Study site

The Flakaliden Experimental Forest (64°07′N, 19°27′E) is situated in the boreal zone of northern Sweden (Ahti et al., 1968). The site consists of a Norway spruce forest (*Picea abies* (L.) Karst) planted in 1963 after clear-felling. The understory layer is dominated by the ericaceous species, *Vaccinium myrtillus* L. and *V. vitis-idaea* L., and to a lesser extent by the grass *Deschampsia flexuosa* and the fern *Gymnocarpium dryopteris* (L.) Newman. The moss layer is dominated by *Hylocomium splendens* (Hedw.) B.S.G and *Pleurozium schreberi* (Bird), and to a minor extent by *Polytrichum commune* Hedw. Soils at the site are Typic Haplocryods (US soil classification system) developed from silty-sandy tills (Olsson et al., 2005). The mean annual precipitation at the site is 645 mm and the mean monthly air temperatures are −7.5 °C and 14.6 °C in February and July, respectively (Sigurdsson et al., 2013). Background atmospheric N deposition in this region is approximately 3 kg N ha^{-1} yr^{-1} (Pihl Karlsson et al., 2011).

2.2. Experimental design

The long-term nutrient optimization experiment was set up in 1987 (Linder, 1995; Bergh et al., 1999) and consists of a randomized block design of three replicates per treatment (50 m × 50 m plots). Each block consisted of three treatments: untreated plots (water irrigation only), a short-term nutrient optimization treatment (SO) started in 2007, and a long-term nutrient optimization treatment (LO) started in 1987. Macro (N: 75–100 kg N ha^{-1} yr^{-1}, P: 10–21 kg P ha^{-1} yr^{-1}, K: 30–48 kg K ha^{-1} yr^{-1}, Ca: 0–53 kg Ca ha^{-1} yr^{-1}, S: 0–8 kg S ha^{-1} yr^{-1} and Mg: 0–18 kg Mg ha^{-1} yr^{-1}) and micro-nutrients (Mn, Fe, Zn, Cu, B, Mo) were annually supplied, dissolved in water. The fertilization treatments were adjusted annually by monitoring spruce needle nutrient concentrations, in an attempt to achieve optimal tree growth, while simultaneously avoiding nutrient leaching from the rooting zone. For further details on the protocol, see Linder (1995). Nitrogen was supplied in the form of liquid ammonium-nitrate (NH_4NO_3). Each nutrient optimization treatment has been applied with a sprinkler system every second day during the growing season between June and mid-August for the whole duration of the experiment. During a single year (2013), we used this experimental system to make a variety of measurements to describe litter and microbial stoichiometry as well as soil food web structure. While our focus on a single year limits our ability to infer inter-annual variability of responses, the long duration of the experiment provides a powerful tool to reveal relative treatment differences that may have emerged.

2.3. Litter and soil parameters

In October 2013, we collected intact litter samples from the forest floor (Oi horizon) at ten random locations using a quadrat (0.25 m^2) for each plot. All samples were pooled together to obtain one composite sample per plot. The litter was sorted and allocated to three categories: spruce needles, twigs and deciduous tree leaves, as these were the most abundant litter types encountered when sampling. The litter samples were dried (60 °C for 72 h) and used for chemical characterization. Litters were ground using a ball mill (Retsch MM 301; Haan, Germany). We analyzed total C and N litter content by dry combustion using an elemental analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany), and total P content using the Kjeldahl method and an auto-analyzer III (SEAL Analytical, OmniProcess AB, Sweden) (Maaroufi et al., 2016). We also measured the soil pH of the organic horizon (see Section 2.4 for further details) by using 10 mL air dried soil and 30 mL of deionized water, shaken during one hour and measured at 20 °C.

2.4. Microbial biomass and community structure

Soil microbial communities were analyzed using the microbial phospholipid fatty acids method (PLFA). In October 2013, four soil core samples (10 cm diameter) were randomly collected from each plot spanning the organic horizon (Oi, Oe, and Oa sub-horizon combined, US soil classification system) while excluding the mineral soil horizon. The soil cores were transferred to sealed plastic bags and stored at 4 °C prior to sieving. The soil was sieved on a 4 mm sieve to remove roots, stones and green plant fragments, and the four samples were then pooled to obtain a composite sample per plot. After sieving, the samples were freeze-dried. PLFAs were extracted from two 0.3 g (dry mass) subsamples of each soil sample using a modified method of Bligh and Dyer (Bligh and Dyer, 1959; White et al., 1979). The abundance of PLFAs was quantified using a Perkin Elmer Clarus 500 gas chromatograph (Waltham, Massachusetts, USA), and was converted to μmol PLFA per g organic matter using conventional nomenclature (Frostegård and Bååth, 1996; Tunlid et al., 1989). Bacterial PLFAs including i-15:0, α-15:0, 15:0, i-16:0, 16:1ω7, 16:1ω9, 16:0, i-17:0, cy-17:0, cy-19:0, α-17:0, 18:1ω7 were used as indicators of total bacterial biomass, while the PLFA 18:2ω6,9 was used as an indicator of fungal biomass. The branched fatty acids 10me16:0, 10me17:0 and 10me18:0 were used as indicators of actinomycete biomass. The branched fatty acids i-15:0, α-15:0, i-16:0, i-17:0 and α-17:0 were used as indicators of gram-positive bacteria biomass, while cy-17:0, cy-19:0, 16:1ω7 and 18:1ω7 were used as indicators of gram-negative bacterial biomass. We also calculated the relative abundance (%) of each microbial group.

2.5. Microbial stoichiometry

Soil microbial C, N, and P were measured using the chloroform fumigation extraction method (Beck et al., 1997; Brookes et al., 1985) with 20 g of moist soil for each of the fumigated and non-fumigated treatments. The fumigated and non-fumigated soils were extracted with 75 mL 500 mM K_2S0_4, placed for one hour on a shaker, and filtered onto precombusted Whatman glass microfibre filters (GF/F). The extracts were then frozen until analysis. Organic C and N in the extracts were measured using a Hach-Lange IL-550 analyzer (Hach-Lange, Germany), while P was measured using a FIA star 5000 analyzer (FOSS Tecator, Höganäs, Sweden). Microbial biomass was calculated as follows: biomass C = E_C/k_{EC}, where E_C is (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil), and k_{EC} is 0.45 for C (Beck et al., 1997). The same formula was used to calculate biomass N and P with k_{EN} = 0.54 (Brookes et al., 1985), and k_{EP} = 0.40 (Brookes et al., 1982; Turner et al., 2001). All biomasses were expressed in mg per kg soil.

2.6. Nematodes

Free-living soil nematodes were extracted using the centrifugation method (Oostenbrink, 1960). In October 2013, ten cylindrical soil cores (2 cm diameter) spanning the organic horizon were equidistantly collected from each plot, pooled and transferred to sealed plastic bags, then stored at 4 °C prior to nematode extraction. Briefly, soil samples were gently homogenised by hand and successively wet sieved through 180 µm and 38 µm sieves. The sieved soil solution was successively centrifuged (3000 rpm), the supernatant was discarded, and a sucrose solution was added to the solution followed by centrifugation (3000 rpm) for two minutes. The supernatant was sieved (25 µm) and the final solution collected for nematode fixation. The nematodes were heat-killed and fixed using a solution of 4% formaldehyde (at 60 °C). A minimum of 300 nematodes were identified to family or genus level according to Bongers (1988) and assigned to the following five feeding groups: plant-feeders, bacterial-feeders, fungal-feeders, omnivorous, and carnivorous nematodes (Yeates et al., 1993). The Maturity Index (MI) was calculated using the colonizer-persister classification which is an indicator of the disturbance state of the nematode fauna (Bongers, 1990; Bongers and Bongers, 1998). Each nematode taxon was also allocated to functional guilds (*i.e.* assemblage of species with similar biological and ecological characteristics) (Ferris et al., 2001) to be able to calculate the Channel Index (CI), which indicates the predominant decomposition pathways (*i.e.*, fungal *versus* bacterial). Further, we calculated the Enrichment Index (EI), which indicates the abundance of opportunistic bacterial and fungal feeders (Ito et al., 2015). The family richness and the Shannon-Wiener diversity index (Ĥ) were also calculated (Spellerberg and Fedor, 2003). The nematode abundance for each taxon was expressed in number of nematodes per 100*g* of dry soil. We also calculated the relative abundance (%) of each nematode feeding group.

2.7. Micro-arthropods

Soil micro-arthropods were extracted using modified Tullgren extractors (Van Straalen and Rijninks, 1982). In October 2013, five cylindrical soil samples (10 cm diameter, 10 cm depth) were randomly taken from each plot of each treatment (n = 3), transferred to sealed plastic containers and directly placed in the Tullgren extractor for 3 weeks. Micro-arthropods were collected into vials containing 70% ethanol. Present Araneae (spiders) were counted. Collembola were identified to the species level according to (Fjellberg, 1998, 2007), and assigned to three functional groups: eu-edaphic (living in the soil), hemi-edaphic (living in the litter layer) and epi-edaphic (living on the ground surface) (Bokhorst et al., 2012). Acari were identified to three suborder levels: Mesostigmata, Oribatida, and Astigmata-Prostigmata according to Krantz and Walter (2009). When possible, Oribatida were identified to the family level according to Weigmann (2006). The Oribatida were classified as fungivores (Smith and Scudder, 1998), while the Mesostigmata were classified as predators (Gan et al., 2013; Taylor et al., 2010) (see Fig. 1). We calculated the abundance, the Shannon diversity Index (Ĥ) as well as the richness, for Collembola and Acari at the species level and suborder level, respectively. We also calculated the relative abundance (%) of Collembola functional groups and Acari suborders.

2.8. Statistical analysis

All response variables were tested for normality and homoscedasticity prior to statistical analyses. Data were transformed using [Log (x + 1)] or [√(x + 0.5)] when these assumptions were not met. Litter quality parameters, PLFA data, microbial biomass data, Araneae abundance, nematodes (*i.e.* total, family and functional group abundance, family richness, Ĥ), Collembola (*i.e.* total, individual species, functional group abundance, species richness and Ĥ), Acari (*i.e.* total and family/order abundance, family/order richness and Ĥ) data were tested to compare differences among nutrient optimization treatments using Linear Mixed Models with nutrient optimization treatment used as a fixed factor and block as a random factor. When significant differences between treatments were detected ($\alpha = 0.05$), *post hoc* pairwise comparisons between treatments were conducted using the Student-Newman-Keuls test.

We performed permutational multivariate analysis of variance (PERMANOVA; $\alpha = 0.05$) to determine whether multivariate differences occurred in response to nutrient optimization treatments on PLFA markers, nematode families, Collembola species and Acari suborders (Gundale et al., 2016). We also determined Bray-Curtis dissimilarities for each community and we performed nonmetric multidimensional scaling (NMDS) using relative abundance data for micro- and

Table 1
Mean and ± SE of carbon, nitrogen and phosphorus (%) and associated molar ratios for *Picea abies* litter needles, twigs and deciduous leaves in response to nutrient optimization treatments: control, short-term (since 2007) and long-term (since 1987). The F- and P-values were derived from one-way ANOVAs (the degrees of freedom are 2,9 except for *P. abies* twigs %P, C:P and N:P (the degree of freedom are 2,6) due to the lack of ground material). Different letters (a or b) indicate significant differences between treatments determined using Student-Newman-Keuls *post hoc* tests. Values in bold indicate statistical significances at $P < 0.05$.

	Control		Short-term		Long-term		*F*-value	*P*-value
P. abies needles								
C:N	49.95**b**	± 1.07	38.33**a**	± 1.11	37.98**a**	± 1.31	34.02	**0.001**
C:P	1173.33**b**	± 32.48	691.85**a**	± 12.18	670.60**a**	± 16.48	164.43	< **0.001**
N:P	23.48**b**	± 0.23	18.06**a**	± 0.31	17.68**a**	± 0.41	100.30	< **0.001**
%C	51.06**a**	± 0.28	51.99**b**	± 0.26	52.66**b**	± 0.09	12.98	**0.007**
%N	1.19**a**	± 0.02	1.59**b**	± 0.05	1.62**b**	± 0.05	26.84	**0.001**
%P	0.11**a**	± 0.00	0.19**b**	± 0.00	0.20**b**	± 0.01	125.60	< **0.001**
P. abies twigs								
C:N	59.01	± 4.87	60.11	± 0.39	51.52	± 1.31	1.25	0.379
C:P	1570.58	± 107.91	–	–	1279.23	± 7.78	4.37	0.128
N:P	26.72	± 1.14	–	–	24.85	± 0.48	1.54	0.303
%C	54.28**b**	± 0.06	50.11**a**	± 0.03	55.74**c**	± 0.16	938.00	< **0.001**
%N	1.09	± 0.09	0.97	± 0.01	1.26	± 0.03	3.13	0.152
%P	0.09	± 0.01	–	–	0.11	± 0.00	7.20	0.075
Deciduous leaves								
C:N	31.11	± 3.09	35.77	± 2.94	40.14	± 8.56	0.49	0.642
C:P	587.68**b**	± 9.42	583.20**b**	± 16.24	432.22**a**	± 14.18	36.89	**0.001**
N:P	19.05**b**	± 1.59	16.44**ab**	± 0.87	11.52**a**	± 1.80	6.46	**0.041**
%C	52.20	± 0.16	52.49	± 0.69	52.84	± 0.31	0.36	0.713
%N	1.98	± 0.20	1.74	± 0.15	1.67	± 0.28	0.46	0.655
%P	0.23**a**	± 0.01	0.23**a**	± 0.01	0.31**b**	± 0.01	33.32	**0.001**

mesofauna and microbial PLFA biomasses to describe community differences among nutrient optimization treatments.

We further performed correlation analyses between litter nutrient ratios and microbial nutrient ratios using Pearson correlation tests ($\alpha = 0.05$). We performed correlation analyses between primary consumer (microbe) nutrient stoichiometry and their food resource quality in terms of litter C:N, C:P and N:P ratios (Fig. 1). P-values were corrected for multiple testing using the Benjamini and Hochberg false discovery rate correction (Verhoeven et al., 2005).

All univariate and correlation analyses were performed using SPSS (Chicago, Illinois, USA; v. 20.0), while the multivariate analyses were performed using CANOCO (Biometris, Wageningen, NL; v. 5.0).

3. Results

3.1. Litter stoichiometry and soil parameters

Both short- and long-term nutrient optimization treatments significantly affected litter nutrient ratios for the three litter types (Table 1). For *P. abies* needles, the %C, %N and %P significantly increased, while litter C:N, C:P and N:P ratios significantly decreased for both treatments relative to the control. We found that the %C was significantly increased by 1.8% and by 3.0% in the short- (SO) and long-term (LO) treatments relative to the control plots. Further, we found that the %N was significantly increased by 25.2% and by 26.5% in the SO and LO treatments relative to the control plots. The %P was significantly increased by 42.1% and by 47.4% in the SO and LO treatments relative to the control plots. The litter C:N was significantly decreased by 23.3% and by 23.9% in the SO and LO treatments relative to the control plots. The C:P was significantly decreased by 41.0% and by 42.9% in the SO and LO treatments relative to the control plots. The N:P ratio was significantly decreased by 23.1% and by 24.7% in the SO and LO treatments relative to the control plots. No significant effects were found for *P. abies* twigs, except that %C was significantly decreased by 7.7% in the SO treatment, while it increased by 2.6% in LO treatments relative to the control plots. For deciduous leaf litter, we found a significant increase in %P by 25.8% and a decline in the C:P ratio by 26.5% in the LO treatment relative to the control. Finally the deciduous leaf litter N:P ratio significantly declined by 25.8% in the LO treatment relative to the control. The optimization treatments had no effect on soil pH ($F = 3.20$, $P = 0.113$).

3.2. Microbial PLFA structure, biomass and stoichiometry

PLFA marker composition significantly differed among nutrient optimization treatments (Fig. 2a, Table S1). Composition of the two treatments significantly differed from the control but not between each other (see bottom right corner of Fig. 2a). PLFA markers contributing to the similarities and differences between treatments are reported in Tables S2–S5. Briefly, the 18:2ω6 fungal, the 18:1ω9 general and 16:0 bacterial PLFA markers altogether contributed to 55.2% and 59.7% of the dissimilarities between the control and the SO and LO treatments, respectively.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foreco.2018.06.034.

Comparisons of the PLFA functional group biomasses using ANOVA showed significant differences for total PLFAs, bacterial, and fungal functional groups (Fig. 3a). In each of these cases, the PLFA markers in

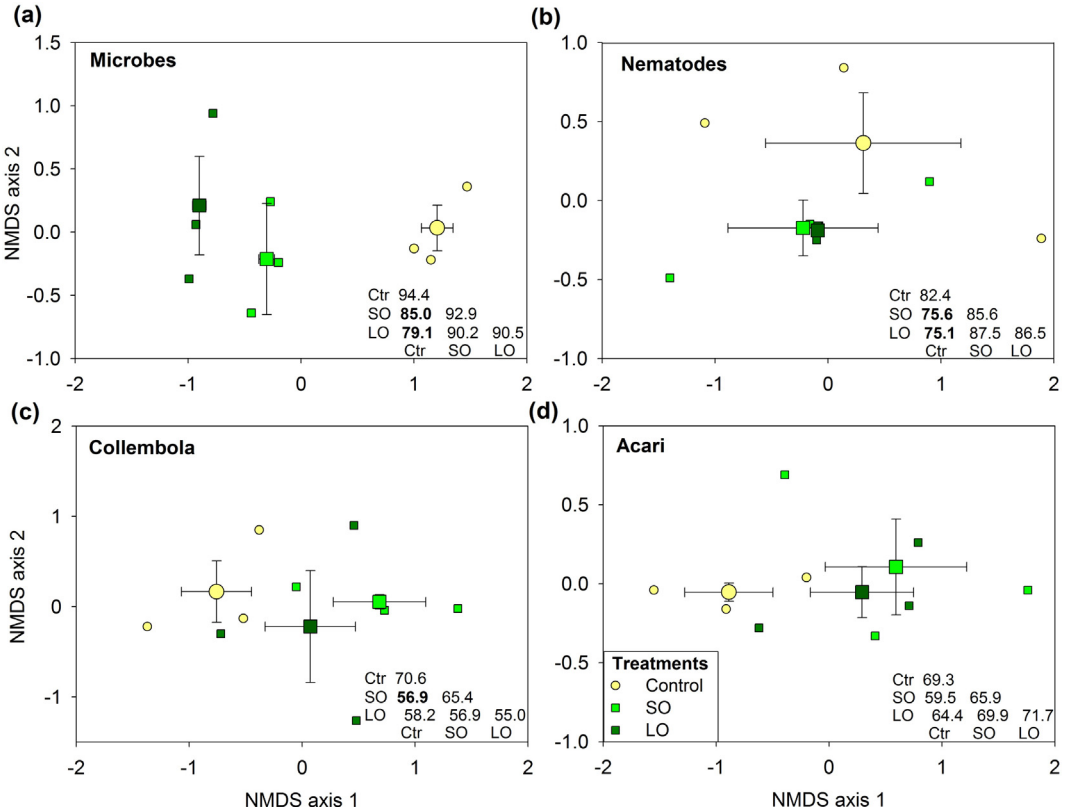

Fig. 2. Nonmetric multidimensional scaling (NMDS) of microbial phospholipid fatty acid (PLFA) markers (a), nematode families (b), Collembola species (c) and Acari suborders (d). Insets show average of pairwise similarities (%) between and within groups. Bold values indicate significant differences ($P < 0.05$) in community composition based on PERMANOVA (permutations multivariate analysis of variance). Treatments consisted of control (yellow circle, Ctr), nutrient optimization applied since 2007 (light green square, SO), and nutrient optimization applied since 1987 (dark green square, LO). Small symbols correspond to the mean for each plot, large symbols correspond to the mean per treatment. Bars indicate 95% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

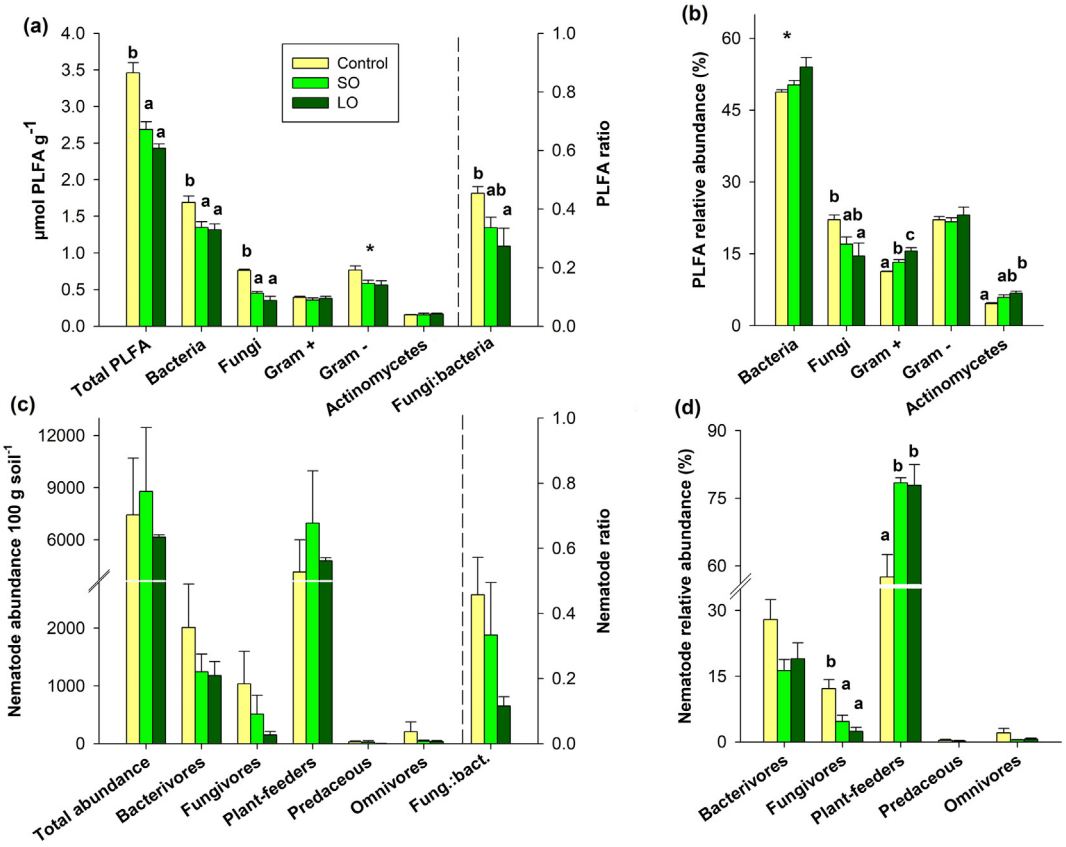

Fig. 3. Mean (+SE) biomass, abundance, ratios (left panels) and relative abundance (right panels) for microbial phospholipid fatty acids (PLFA) (a and b) and nematodes (c and d) in response to nutrient optimization treatments. Treatments consisted of control (yellow, Ctr), nutrient optimization applied since 2007 (light green, SO), and nutrient optimization applied since 1987 (dark green, LO). Different letters (a, b or c) on top of each bar indicate significant differences ($P < 0.05$) between treatments determined using Student-Newman-Keuls post-hoc tests. * indicates marginally significant differences ($0.05 \leq P < 0.1$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the SO and LO treatments significantly declined relative to the control. We found that the total PLFA biomass was significantly reduced by 22.5% and by 30% in the SO and LO treatments relative to the control plots. Bacterial PLFA biomass declined by 20% and by 22% in the SO and LO treatments relative to the control plots. Fungal PLFA biomass declined by 41% and by 54% in the SO and LO treatments relative to the control plots. Further, the fungi:bacteria ratio non-significantly decreased by 24% and significantly decreased by 40% in the SO and LO treatments relative to the control, respectively. There were no significant changes among treatments for the gram-positive, gram-negative and actinomycetes PLFA group abundances.

Comparisons of the relative abundance (%) of PLFA functional groups showed that significant differences occurred for fungal, gram-positive and actinomycete functional groups in response to the nutrient optimization treatments (Fig. 3b). The relative abundance of fungal PLFA was reduced by 34.9% in the LO treatment relative to the control. The relative abundance of gram-positive PLFA was significantly increased by ~27.4% in the LO treatment relative to the control. The relative abundance of actinomycete PLFA was increased by 32.6% in the LO treatment relative to the control.

With the exception of the microbial C:P ratio, the nutrient stoichiometry in microbial biomass was not affected by the nutrient optimization treatments. We found that the microbial C:P ratio was significantly reduced by 41% in both treatments relative to the control plots (Table 2).

Table 2
Mean and ± SE concentrations of carbon, nitrogen and phosphorus (mg kg^{-1}) and associated molar ratios for microbial biomass in response to nutrient optimization treatments: control, short-term (since 2007) and long-term (since 1987). The F- and P-values were derived from one-way ANOVAs (the degrees of freedom are 2,9). Different letters (a or b) indicate significant differences between treatments determined using Student-Newman-Keuls *post hoc* tests. Values in bold indicate significant differences at $P < 0.05$.

	Control		Short-term		Long-term		*F*-value	*P*-value
Microbial biomass								
C biomass (mg kg^{-1})	2224.82	84.35	1514.52	301.49	1724.65	450.70	1.33	0.333
N biomass (mg kg^{-1})	65.20	3.15	50.02	10.94	65.57	21.68	0.54	0.607Ɨ
P biomass (mg kg^{-1})	99.64	10.61	84.91	9.11	126.69	27.54	1.41	0.314
Molar ratio								
C:N	39.88	± 2.19	31.99	± 4.07	35.62	± 3.98	3.77	0.087
C:P	58.70**b**	± 8.38	34.78**a**	± 4.14	34.84**a**	± 1.93	15.01	**0.008**
N:P	1.48	± 0.27	1.11	± 0.26	0.96	± 0.08	3.24	0.125

Ɨ the data were transformed using the function log(x + 1) to meet the assumption of normality and variance.

Table 3
Mean and ± SE of richness, Shannon-Wiener index (Ĥ) for micro- and meso-fauna as well as the nematode Maturity Index (MI), the Channel Index (CI) and the Enrichment Index (EI) in response to nutrient optimization treatments: control, short- (since 2007) and long-term (since 1987). The F- and P-values were derived from a one-way ANOVA for each variable. Different letters (a or b) significant differences between treatments determined using Student-Newman-Keuls *post hoc* tests. Values in bold indicate statistical significances at $P < 0.05$. § Kruskal-wallis (df 2; chi-square = 3.96) *block used as random factor.

	Treatments							
	Control		SO since 2007		LO since 1987		*F*-value	*P*-value
Nematodes								
Richness	15.67	± 0.88	17.33	± 0.33	17.33	± 0.33	3.96	0.138§
Ĥ	1.48**b**	± 0.08	1.09**a**	± 0.08	1.07**a**	± 0.16	7.76	**0.042***
MI	2.08**b**	± 0.00	2.05**ba**	± 0.01	2.02**a**	± 0.02	8.15	**0.019**
CI	92.54**b**	± 4.08	96.30**b**	± 3.70	72.12**a**	± 3.61	11.71	**0.008**
EI	49.44	± 0.59	49.44	± 1.76	47.49	± 4.61	0.15	0.861
Collembolans								
Richness	7.73	± 0.76	7.60	± 1.06	6.01	± 1.54	0.68	0.543
Ĥ	2.04	± 0.05	2.34	± 0.09	2.04	± 0.10	4.28	0.070
Acari								
Richness	6.87	± 0.13	5.63	± 0.43	6.01	± 0.27	4.32	0.069
Ĥ	1.81	± 0.06	1.70	± 0.06	1.66	± 0.05	1.97	0.219

3.3. Nematodes

PERMANOVA analysis showed that nematode family composition in the two optimization treatments significantly differed from the control but not between each other (see bottom right corner of Fig. 2b). Nematode families contributing to the similarities and differences between treatments are given in Tables S6–S9. Briefly, Tylenchidae (plant feeders), Plectidae (bacterial feeders), Aphelenchoidae (fungal feeders) and Criconematidae (plant feeders) together contributed to 74.3% of the dissimilarities between the control and the SO treatment. Tylenchidae (plant feeders); Plectidae, Aphelenchoidae and Bunonematidae (all bacterial feeders) together contributed to 75.3% of the dissimilarities between the control and the LO treatment. The Shannon-Wiener index (Ĥ; mean ± SE) declined in response to both the SO (1.09 ± 0.1) by ~26% and in the LO (1.07 ± 0.2) by ~28% treatments relative to the control (1.48 ± 0.1) (Table 3).

Nematode family (Table S10) and feeding group abundances were not significantly affected by nutrient optimization treatments. However, fungal feeders were relatively less abundant and plant feeders were relatively more abundant under fertilization (Fig. 3c and d, Table S1). The Maturity Index (mean ± SE) declined by 3% in the LO treatment (2.02 ± 0.0) relative to the control (2.08 ± 0.0) (Table 3). The Channel Index declined by 22% in LO (72.1 ± 3.6) relative to the control (92.54 ± 4.1). The Enrichment Index was not affected by the nutrient optimization treatments.

3.4. Micro-arthropods

PERMANOVA analyses showed that only Collembola species composition from the SO treatment significantly differed from the control (Table S1, left corner of Fig. 2c), while the Acari taxon composition did not differ between optimization treatments and the control (Fig. 2d, Table S1). Collembola species contributing to the similarities and differences between treatments are given in Tables S11–S14. Briefly, *Protaphorura* sp. (eu-edaphic), *Parisotoma notabilis* (hemi-edaphic), *Isotomiella minor* (eu-edaphic) and *Tullbergia* sp. (eu-edaphic) together contributed to about 60% of the dissimilarities between the control and the SO treatment.

ANOVA analyses on the abundance of hemi-edaphic Collembola, two individual epi-edaphic species, one Oribatida family and Araneae showed significant differences among optimization treatments (Fig. 4, Table S15). Hemi-edaphic Collembola abundance was non-significantly increased by 26% and significantly increased by 40% in the SO and LO treatments relative to the control plots, respectively (Fig. 4a). The epi-edaphic Collembola *Lepidocyrtus lignorum* abundance declined by 78.5% and by 67.1% relative to the control in the SO and LO treatments, respectively (Table S15). The epi-edaphic Collembola *Pogonognathellus flavescens* abundance declined by 95.3% in the LO treatment. The relative hemi-edaphic abundance significantly increased by 49.8% in response to SO treatment relative to the control (Fig. 4b, Table S1). Araneae abundance declined by 33.7% and by 74.0% relative to the control in the SO and LO treatments, respectively. The nutrient optimization treatments had no significant effect on total Acari abundance and relative abundances (Fig. 4c and d), except for Nothridae (oribatid mite) which non-significantly decreased by 52.2% and significantly decreased by 100% relative to the control in the SO and LO treatments, respectively (Table S15).

3.5. Stoichiometric relationships

Correlation analysis revealed no significant correlation between microbial nutrient and litter nutrient stoichiometry (Table S16).

4. Discussion

Our aim was to investigate the short- and long-term impacts of nutrient optimization treatments on soil biota and the relationships between plant litter stoichiometry and microbial nutrient stoichiometry in a boreal forest.

In support of our first hypothesis, the total microbial, fungal and bacterial PLFA biomasses declined in response to both short- and long-term nutrient optimization treatments. Also in support of our first hypothesis, the microbial community structure was significantly different in the short- and long-term treatments compared to the control plots, with the fungal PLFA 18ω6 marker being a particularly important contributor to the microbial community dissimilarities between the control and the nutrient optimization treatments. Further, the fungi:bacterial ratio also differed between the long-term optimization treatment relative to the control, indicating that fungal biomass was more affected by the long-term treatments than bacterial biomass. These responses to nutrient optimization are similar to patterns that have been reported in studies that fertilized with only N (Maaroufi et al., 2015; Treseder, 2008). One mechanism that may explain the decline in soil fungal biomass in both of these study types is that enhanced nutrient availability may cause plants to reduce C allocation belowground, which in turn would reduce C supply to mycorrhizal fungi (Litton et al., 2007; Ryan, 2013). Previous studies conducted in our nutrient optimization experiment have shown that nutrient optimization causes an increase in tree stem wood (Bergh et al., 2005; Sigurdsson et al., 2013) and a corresponding decline of soil respiration (Olsson et al., 2005),

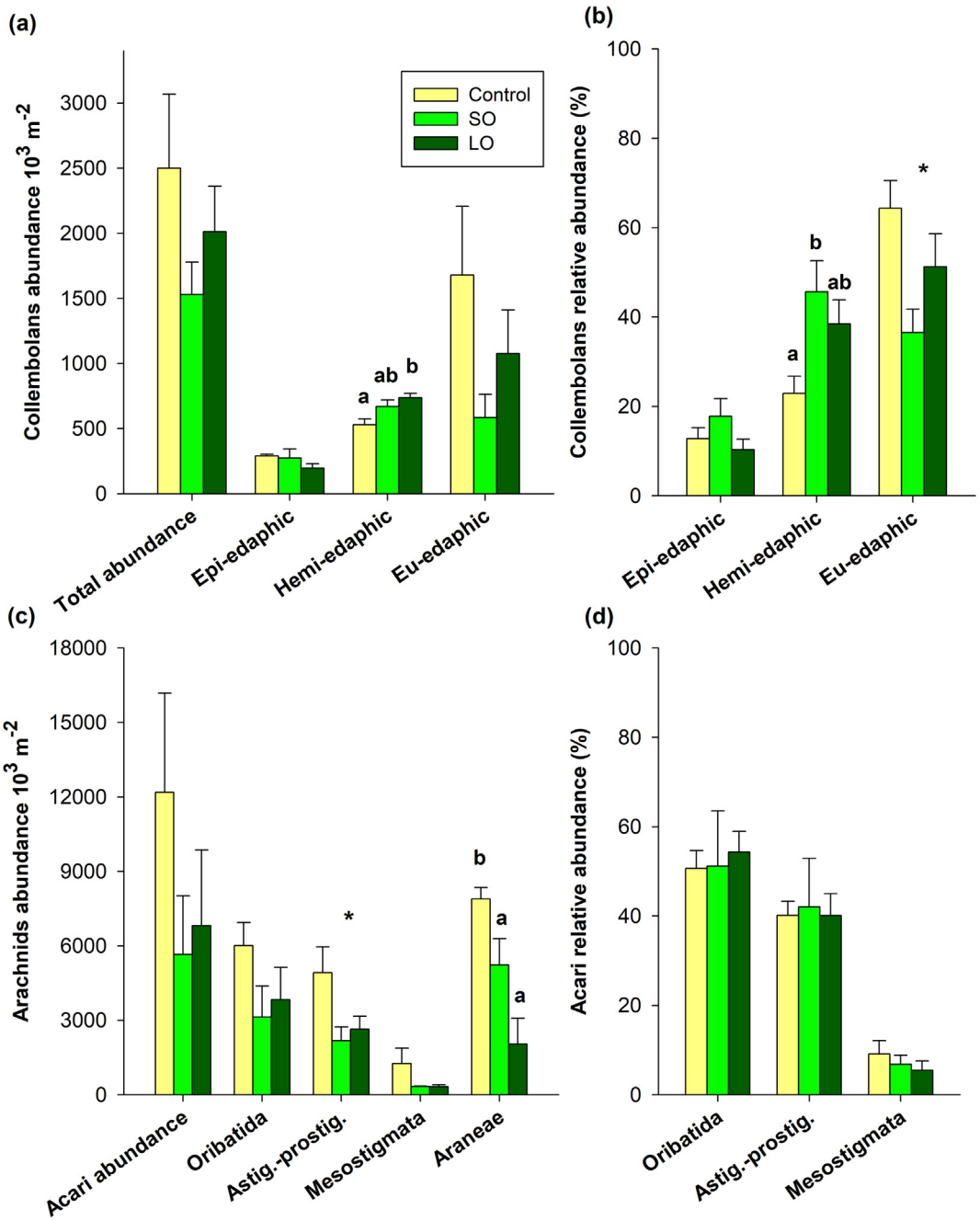

Fig. 4. Mean (+SE) abundance (left panels) or relative abundance (right panels) for Collembola (a and b) or arachnids (c and d) in response to nutrient optimization treatments. Treatments consisted of control (yellow, Ctr), nutrient optimization applied since 2007 (light green, SO), and since 1987 (dark green, LO). Different letters (a or b) on top of each bar indicate significant differences ($P < 0.05$) between treatments determined using Student-Newman-Keuls post-hoc tests. * indicates marginally significant differences ($0.05 \leq P < 0.1$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting increased aboveground C allocation at the expense of belowground allocation (Ryan, 2013). Similarly, N-only addition studies have reported a decline of soil respiration, especially when N is applied in high doses similar to our nutrient optimization treatments (≥ 50 kg N ha^{-1} yr^{-1}) (Hasselquist et al., 2012; Janssens et al., 2010) as well as increased tree growth (From et al., 2016; Gundale et al., 2014), and reduced fungal biomass (Högberg et al., 2010).

Inconsistent with our second hypothesis, the proportion of C, N, and P in microbial biomasses and their C:N and N:P ratios were not affected by the nutrient optimization treatments. These findings indicate that, despite changes in plant litter stoichiometry in response to nutrient optimization, C:N and N:P microbial stoichiometry remained stable. Our results contrast previous studies that showed that microbial C:N:P stoichiometry can vary with its surrounding environment (Fanin et al., 2013; Tischer et al., 2014). Instead, our findings support the common view that stoichiometry of microbial communities is globally very stable and independent of the environmental nutrient availability. Previous studies have suggested that microbial C:N ratios may remain stable as N is usually not stored long-term but rather metabolized, mineralized or excreted by microbial cells (Banham and Whatley, 1991; Mooshammer et al., 2014).

However, in line with our second hypothesis, we found that the C:P ratio of microbial biomass declined in response to both nutrient optimization treatments. However, microbial C:P ratio was not correlated

to the corresponding litter resource C:P ratios. An explanation may be that microbial community structure shifted towards community members with lower C:P ratios which may have altered the microbial stoichiometry (Fanin et al., 2013; Heuck et al., 2015). For example, a shift towards a higher proportion of bacteria compared to fungi could have reduced microbial community C:P ratios, since bacteria have a ca. three-fold higher concentration of P-rich nucleic acids relative to the total cell mass than fungi (Kirchman 2012 and references therein). This mechanism is supported by the decline of the fungi:bacterial ratio in the long-term nutrient optimization treatment in our study. Another possible mechanism that has been proposed is that soil microbes may take up P in excess, and store it as polymers (*e.g.* glycogen, polyphosphates) resulting in a higher C:P ratio (Achbergerová and Nahálka, 2011; Mooshammer et al., 2014).

We found only partial support for our third hypothesis as most organisms belonging to the fungal and bacterial feeding energy channels were rather unresponsive to the nutrient optimization treatments. These results are in contrast with Berch et al. (2009), who found an increase in fungivorous and predaceous mites after 10 years of macro- and micro-nutrient additions in a sub-boreal spruce forest. Nitrogen-only addition studies reported a decline of the Acari abundance in northern and sub-alpine forests after 12–17 years of N additions (Gan et al., 2013; Xu et al., 2009). Comparison among studies are difficult as each of them have focused on the addition of one nutrient or different combinations of nutrients. While most bacterial and fungal feeders were unresponsive, we did find that hemi-edaphic Collembola, which live in the litter layer, decreased in the long-term nutrient optimization treatment. Further, we observed a decline of Araneae abundance (which are predators of Collembola) in response to both optimization treatments, which may be due to a reduction in their prey. Our results are in contrast with Lindberg and Persson (2004) who reported an increase in abundance of Collembola in response to nutrient optimization which they suggested was due to a bottom-up effect on the quality of litter entering the soil.

In support of our third hypothesis, nematode indices describing the state of the nematode food web were responsive to the long-term nutrient optimization treatment. We found a decrease of the Channel Index in the long-term treatment indicating a shift towards a more bacterial-dominated decomposition pathway. Further, we found a lower Maturity Index in the long-term treatment, indicative of soil disturbance, and an increase in the proportion of colonizer nematodes (r-strategists) that have a higher tolerance to soil eutrophication than persister nematodes (K-strategists). However, inconsistent with our third hypothesis, the nematode feeding group abundances showed only downward trends in response to nutrient optimization, suggesting that nematode functional guilds (*i.e.* used to calculate the indices and based on feeding groups and life strategies) rather than feeding groups themselves reflected shifts in nematode communities in response to nutrient optimization. Our results are in line with Cesarz et al. (2015) who showed that nematode functional guilds were better soil indicators than nematode feeding groups for food web responses to N addition.

We demonstrated that long-term nutrient optimization that aims to increase forest production (*e.g.* timber volume, biofuels) or enhance sequestration of anthropogenic CO_2 emissions causes a decrease of all litter nutrient ratios, the microbial C:P ratio and soil fungal biomass. The decrease in soil fungi may be due to a decline of tree belowground C allocation, which may explain the lower fungi:bacterial ratios we observed in response to nutrient optimization. In contrast, nutrient optimization had contrasting impacts on soil organisms higher up in the soil food web. The shifts in soil food webs in response to nutrient optimization we report may have important implications for how soils function. For example, reductions in the fungal-based energy channel may lead to the accumulation of recalcitrant organic detritus that fungal enzymatic systems are most efficient at breaking down (*e.g.* lignin) (Eisenlord et al., 2013). Likewise, an increase in bacterial relative to fungal energy channels may lead to reduced N immobilization into fungal biomass, which in turn might result in higher rates of N losses through leaching, denitrification, or N_2O emissions (De Vries et al., 2011; Pilegaard, 2013). Understanding how altered soil food webs in response to nutrient optimization impact ecosystem functions remains a frontier that requires further investigation.

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